The low levels of circulating Doublecortin-like kinase 1 Levels in Breast Cancer

Ozturk N[1], Karadeniz E[2], Duranyildiz D[3], Ozturk G[2], Serilmez M[3], Soydinc HO[3], Akcay MN[2], Ozdemir Y[1], Kara S[2]

[1]Nurinnisa Ozturk, MD, Prof. Correspondence:

[2]Erdem Karadeniz, MD, Assoc Prof.

[3]Derya Duranyildiz, MD, Prof.

[2]Gurkan Ozturk, MD, Prof.

[3]Murat Serilmez, MD, Assist. Prof. Dr.

[3]Hilal Oguz Soydinc, MD, Assoc Prof.

[2]Mufide Nuran Akcay, MD, Prof.

[1]Yakup Ozdemir, Assist. Prof. Dr.

[2]Salih KARA, MD. Assoc Prof.

[1]Ataturk University, Medical School, Department of Biochemistry

[2]Ataturk University, Medical School, Department of General Surgery

[3]Istanbul University, Oncology Institute

This study was founded by Ataturk University Scientific Research Projects Co-ordinating Department (Project Number: TSA-2017-6182)

IRB reference date/number 09.06.2017/25

Correspondence:

Assoc. Prof. Nurinnisa Ozturk, MD

Ataturk University School of Medicine

Department of Biochemistry

25080 Erzurum/Turkey

nurinnisa.ozturk@gmail.com

ABSTRACT

Purpose: Breast cancer is the most prevalent form of cancer among women worldwide, and is a complex disease. The development of biomarkers for early diagnosis is a promising avenue for improving the treatment of breast cancer. Doublecortin-like kinase 1 (DCLK1) is a member of the protein kinase superfamily and doublecortin family. It has been hypothesised that cells expressing DCLK1 in these tumours exhibit stem cell characteristics, suggesting a potential association with tumour initiation and mortality. The objective of this study was to evaluate the serum protein and mRNA levels of the DCLK1 molecule in patients diagnosed with breast cancer.

Methods: The present study comprised 62 female patients diagnosed with breast cancer and 30 healthy controls. DCLK1 serum level was determined by ELISA, and mRNA level was determined by quantitative RT-PCR method before any treatment.

Results: Serum DCLK1 level was found to be statistically significantly lower in breast cancer patients (5.38 ± 1.86 ng/mL) than in the control group (7.5 ± 1.15 ng/mL) ($p = 0.001$). Furthermore, we determined that serum DCLK mRNA expression level was significantly lower in breast cancer patients (0.012-0.09, mean rank: 34.48) compared to the control group (0.04-0.09, mean rank: 61.22) ($p < 0.001$, Z: -4.29).

Conclusion: DCLK1 may be a useful marker for detection of the clinical behaviour of breast cancer.

KEY WORDS: Breast cancer, DCLK1, DCLK mRNA expression

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women worldwide and also is a clinically, morphologically and molecularly complex disease. This heterogeneity cannot be explained by clinical parameters such as tumor size, lymph node status, histological grade and age, or biomarkers such as estrogen, progesterone, and epidermal growth factor receptors that are used in routine diagnosis and follow-up (1). Therefore, studies on breast cancer in recent years have focused on the molecular field. Today, many studies investigate the relationship between DNA (methylation, chromosomal copy number changes and somatic mutations), RNA (miRNA and mRNA expressions) and protein (protein and protein-phosphorus expression) levels and breast cancers. Therefore, breast cancer management is largely related to the diagnosis and specific features of the tumor such as hormone production and genetic mutations (2).

Doublecortin-like kinase-1 (DCLK1, formerly known as DCAMLK1) is a member of the protein kinase superal and doublecortin family. This protein is a C-terminal serine / threonine protein kinase domain that binds to microtubules and regulates microtubule polymerization, the C-terminal serine / threonine protein kinase domain, which is very similar to Ca-calmodulin-dependent protein kinase, and the serine / Contains a proline rich region (3,4). The functions of this protein related to neuronal migration, neuronal apoptosis and neurogenesis were first determined and then its expression was determined in solid tumors such as colon, pancreas, oesophagus and liver, and the tumor was reported as a stem cell marker(3–6). It has been suggested that cells expressing DCLK1 in these tumors show stem cell characteristics and this molecule is associated with tumor onset and mortality. In addition to studies in which there is aberrant DCLK1 expression in invasive breast cancers, interestingly, in a study in which DCLK1 expression was determined immunochemically, it was reported that this molecule was not related to the stem cell properties and aggressive behavior of the tumor (6).

A biomarker that can be used in the diagnosis of breast cancer has not yet been identified. There are studies in the literature on DCLK1 tumor tissue

expression in breast cancer and its use in prognosis evaluation, but there is no study evaluating serum levels for diagnostic purposes. In this study, we aimed to determine and evaluate both protein and mRNA levels of the DCLK1 molecule in patients diagnosed with breast cancer.

PATIENTS AND METHODS

Patient and control groups were composed entirely of women. Our study was carried out prospectively in 62 female patients who were operated on for breast cancer in the General Surgery Clinic of Ataturk University Health Research and Application Center Hospital and 30 healthy controls who have the same demographic characteristics as the control group. Patients receiving neoadjuvant chemotherapy were excluded from the study. Informed consent was obtained from the participants in accordance with the Helsinki declaration and Ataturk University Institutional Review Board guidelines (IRB reference date/number 09.06.2017/25). Blood samples were taken from patients and controls using vacutainer tubes. Samples were kept for 30 minutes to coagulate at room temperature and serum were separated by centrifugation under appropriate conditions (4000 rpm for 10 minutes) and aliquoted and stored at -80 ° C.

Serum DCLK1 Levels Measurement

Serum levels of DCLK1 are measured in the Medical Biochemistry Laboratory of Ataturk University Health Research and Application Center Hospital, using a commercial ELISA kit (Elabscience cat No: E-EL-H0367, USA), by following the experimental stages in accordance with the protocol suggested by the kit manufacturer, and using the Dynex brand automatically. Measured on an ELISA reader device (Dynex Technologies Headquarters, Chantilly, USA).

Serum DCLK1 mRNA Quantification

Serum DCLK1 mRNA levels were determined by quantitative Real-Time Polymerase chain reaction (RT-PCR) method at the Biochemistry Laboratory of Istanbul University Oncology Institute.

Circulating cell-free RNA was extracted using monophasic phenol and guanidine thiocyanate solutions (Roche Diagnostics, Mannheim, Germany). In the preliminary preparation, 200 µL serum and 200 µL chloroform were added to 800 µL RNA isolation solution and left to incubate on ice for 5 minutes with mixing. It was centrifuged at 11000 rpm for 15 minutes at 4 ° C. The RNA phase was placed in a new tube containing 550 µL propanol and centrifuged again (10 min at 4 ° C 11000 rpm). The RNA containing pellet was washed with 75% alcohol and dried at room temperature. Dissolved in RNAase free-water. CDNA synthesis from total RNAs was performed using a commercially purchased cDNA synthesis kit (SCRIPT cDNA Synthesis Kit, Roche Diagnostics, Mannheim, Germany). 10 µg of RNA was used for cDNA synthesis.

To determine the expression of DCLK1 mRNA in serum, GAPDH was used as an internal control and SYBR Gren as a fluorescent molecule (Roche Diagnostics, Mannheim, Germany). A 100 mer primer was used for the DCLK1 gene. Expression levels were measured on the LightCycler®480 Real-Time PCR System instrument (Roche Diagnostics, Roche Molecular Systems Inc. Menheim, Germany). PCR reaction stages were performed in 45 amplification cycles of 95 ° C for 30 seconds (seconds) denaturation, 30 seconds of hybridization at 59 ° C and 1 second elongation at 72 ° C. Exon-spanning specific primers designed using Universal Probe Library software (Roche, Mannheim, Germany) were used.

In the RT-PCR reaction, the cycle in which the fluorescence level exceeds the threshold that can be measured by the device is called the threshold cycle (Ct). For data analysis, gene expressions were calculated using the comparative Ct method, which is normalized by subtracting the Ct value of the endogenous reference from each RNA (ΔΔCt = (Cttarget - Ctreference) sample - (Cttarget - Ctreference) control and 2-ΔΔCt = 2 - [(Cttarget - Ctreference) sample - (Cttarget

- Ctreferans) control] Two independent RT-PCR experiments were used to calculate the mean values.

Statistical Analysis

SPSS 20 (Chicago, IL, USA) program was used for statistical analysis. The compliance of the parameters to the normal distribution was evaluated with the Kolmogorov-Smirnov test. In comparison of normally distributed serum DCLK levels, independent samples t test (Independent Samples test) was used to compare DCLK1 mRNA expression levels that did not show normal distribution, Mann-Whitney U test. ANOVA and Kruskal-Wallis tests were used for statistical evaluation between clinical groups. A value of $p < 0.05$ was used for statistical significance. Results were given as mean ± standard deviation (SD) and minimum-maximum (min-max).

RESULTS

Mean age of the patients and controls were 53 (33-84) and 52 (30-75), respectively. When grouped according to the TNM system, 9 patients had stage I disease, 19 had stage IIA disease, 15 had stage IIB disease, 14 had stage IIIA disease, and 5 had stage IV disease. Seven patients underwent simple mastectomy, 3 breast conserving surgery, 7 breast conserving surgery + axillary dissection, and 45 modified radical mastectomy. In histopathological examination, 47 patients had invasive ductal carcinoma, 5 had invasive lobular carcinoma, 5 had mixed carcinoma (2 ductal + lobular, 2 ductal + mucinous adeno, 1 patient ductal + papillary), 1 had solid papillary carcinoma, and 1 had metaplastic carcinoma.

Serum DCLK1 levels were found to be significantly lower in the breast cancer (5.38 ± 1.86 ng / mL) group compared to the control group (7.5 ± 1.15 ng / mL) ($p = 0.001$). Serum DCLK1 mRNA expression levels were also significantly lower in breast cancer patients (0.012-0.09, mean rank: 34.48) compared to the control group (0.04-0.09, mean rank: 61.22) ($p < 0.001$, Z: 4.29).

When we grouped the tumor diameter according to the T stage as <2 cm (17 patients), between 2-5 cm (34 patients) and greater than 5 and 5 cm (11 patients), both DCLK1 serum level and mRNA expression levels were different among all three groups. However there was no statistically significant difference (p> 0.05, Table 1 and Table 2).

We found that there was no significant difference between lymph node positive (38 patients) and patients without (24 patients) in terms of both DCLK1 serum levels and mRNA levels (p <0.05, Table 1 and Table 2).

Forty-two patients had grade 1 and 2 tumors, and 20 had grade 3 tumors according the histopathological examination of the specimen. Both serum and mRNA levels of DCLK1 protein levels were not different among groups (p> 0.05, Table 1 and Table 2). Similarly, we found that there was no significant difference in both levels between patients with metastasis (5 patients) and patients without metastasis (57 patients) (p> 0.05, Table 1 and Table 2).

Table 1. Serum DCLK1 mRNA expression levels, min-max values and statistical comparisons in different groups

	Tumor diameter			LN status		Grade		Metastasis	
Grup (n)	<2 cm (17)	2-5 cm (34)	>5 cm (11)	- (24)	+(38)	1- 2 (42)	3 (20)	-(57)	+ (5)
Serum DCLK mRNA	0,01-0,09	0.002-0.09	0.012-0.075	0.01-0.093	0.001-0.099	0.01-0.093	0.002-0.099	0.002-0.093	0.013-0.099
Mean Rank	32.18	33.24	25.09	31.88	31.26	32.19	30.05	31.91	26.80
p- Z	0.422 – 1.727*			0.89- -0.130		0.66- -0.437		0.56- -0.608	

LN: lymph node, *: Chi-Square value. DCLK1: Doublecortin-like kinase 1. DCLK mRNA expression are given in min-max, $2^{-\Delta\Delta Ct}$

Table 2. The mean± SD values of serum DCLK1 levels in and statistical comparisons different groups and statistical comparisons

	Tumor diameter			LN status		Grade		Metastasis	
Serum DCLK 1 levels	5.55±1.9	5.46±1.8	4.92±2.1	5±1.96	5.63±1.8	5.47±1.6	5.22±2.4	5.36± 1.9	5.76±1. 5
p	0.66			0.20		0.62		0.65	

DCLK1: Doublecortin-like kinase 1, Serum DCLK1 level was given as ng/mL, LN: lymph node

Among the laboratory parameters evaluated at the time of diagnosis, lactate dehydrogenase (LDH) levels were found to be above the reference range of 250 U / L (13 patients) and below (49 patients), and CA15-3 values above 32 U / mL (4 patients).) and below 32 U / mL (58 patients), the serum DCLK1 mRNA expression level we evaluated (for 0.001-0.09 <250 U / L and 0.01-0.08 for ≥250 U / L p = 0.94, Z = -0.08) and serum protein levels (5.41 ± 1.90 ng / mL for <250 U / L and 5.28 ± 1.81 ng / mL for ≥250 U / L, p = 0.83) were not significantly different between both patient groups. (p> 0.05).

When breast cancer patients were classified as molecular, 30 patients were classified as luminal A, 22 patients as luminal B, 6 patients as HER2 Positive, and 4 patients as triple negative. Comparison could be made only between Luminal A and luminal B group patients. Serum DCLK1 levels and mRNA expressions were 5.33 ± 1.86 ng / mL for Luminal A and 5.64 ± 1.35 ng / mL for Luminal B, and 0.0012-0.099 for Luminal A, 0.010-0.068 for Luminal B group patients, respectively. There was no statistically significant difference (p> 0.05) for both serum DCLK1 levels and mRNA expressions between the Luminal A and luminal B group patients.

DISCUSSION

The discovery of new transcriptions, signal transduction pathways, changes in tumor cells and factors affecting cell proliferation will lead to the use of new molecules for the diagnosis and treatment of breast cancers. DCLK1 is a microtubule-related protein and has been defined as a tumor stem cell marker (7,8)

. It has been also reported to play an important role in tumorgenesis (9). The DCLK1 is recently investigated as a tumor stem cell marker in many types of human cancer. In our study, we found that serum protein and mRNA levels of the DCLK1 molecule were lower in patients diagnosed with breast cancer than in the control group.

In another study conducted on patients with head and neck squamous cell carcinoma, it was reported that DCLK1 expression was a prognostic biomarker for short survival, and DCLK1 inhibition could be a therapeutic target (10). It has been also suggested that increased DCLK1 tissue expression in different cancers such as pancreatic (11), hepatocellular (12), and esophageal (13) carcinomas may be associated with metastases and poor prognosis.

Studies investigating tissue expression of DCLK1 have stated that it may be a prognostic marker in breast cancer (14,15). Lv Y et al. (15) High levels of DCLK1 and mRNA expression in basal-like breast cancer tissue and cancer cell lines; is reported to be associated with clinical stage, lend node metastasis, distant metastasis and histologic grade, and also is an independent negative prognostic factor for basal-like breast cancer patients. They also stated in the same study that DCKL1 molecule could be a prognostic marker in basal-like breast cancer and should be a potential treatment target (15). It is also reported that the microRNA miR-424-5p was low in basal-like breast cancer tissue and cancer cell lines, and DCKL1 was found to be a binding site for miR-424-5p and miR-424. Furthermore, 5p was reported to be a tumor suppressor microRNA that regulates tumor proliferation, migration and invasion by binding to its functional target, DCKL1 (16).

On the contrary, in another study conducted in invasive breast cancer patients, it was reported that DCKL1 expression was associated with good clinical features such as low histological grade, absence of lymphovascular invasion, absence of fibrotic foci, absence of necrosis, and low pN stage (5). In the same study, they found that DCKL1 expression is a marker showing good prognosis in invasive breast cancer with neuroendocrine differentiation and therefore, unlike

other gastrointestinal cancers (5). We couldn't find any association between DCKL1 serum protein levels and mRNA expression and lymph node metastasis, distant metastasis and histological grade. According to our results, DCKL1 serum levels were not found to be significant in terms of prognostic parameters.

In a study in which specific gene expressions according to serum estradiol levels were investigated in normal and carcinoma breast tissues, DCKL1 gene expression was found to be lower in breast cancer tissues compared to normal tissue (17). In our study, when the patients with breast cancer and the healthy control group were compared in terms of serum DKCL1 levels and mRNA expression, these values were found to be lower in the patient group. Because of the different molecular subtypes of breast cancer and their different clinical course and biological behaviour, we can say that it may be more appropriate to study DKCLK 1 separately in each molecular subtype.

Wang et al. (16) showed that DCLK1 is overexpressed in basal-like breast cancer tissue and may predict poor prognosis. However, Liu et al. (15) reported that DCLK1 breast cancer tissue expression was positively associated with favourable clinico-pathological features and may be a good prognostic factor in breast cancer, especially in invasive breast cancers with neuroendocrine differentiation. These conflicting reports suggest that the precise role of DCLK1 in breast cancer needs further clarification. There are no studies in the literature evaluating DCLK1 serum levels for the diagnosis of breast cancer.

As conclusion, the evaluation of DCLK1 serum levels in different malignant diseases should be confirmed in future prospective studies with large patient and control cohorts regarding its clinical significance and possible diagnostic value.

REFERRENCES

1. Łukasiewicz S, Czeczelewski M, Forma A, Baj J, Sitarz R, Stanisławek A. Breast Cancer-Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies-An Updated Review. Cancers (Basel). 2021 Aug 25;13(17):4287. doi: 10.3390/cancers13174287.

2. Apostolou P, Papasotiriou I. Current perspectives on CHEK2 mutations in breast cancer. Breast Cancer (Dove Med Press) 2017;9:331-5.
3. Westphalen CB, Quante M, Wang TC. Functional implication of Dclk1 and Dclk1-expressing cells in cancer. Small GTPases 2017;8:164-71.
4. Ikezono YU, Koga H, Abe M, Akiba J, Kawahara A, Yoshida T, et al. High expression of the putative cancer stem cell marker, DCLK1, in rectal neuroendocrine tumors. Oncol Lett 2015;10:2015-20.
5. Chandrakesan P, Yao J, Qu D, May R, Weygant N, Ge Y, et al. Dclk1, a tumor stem cell marker, regulates pro-survival signaling and self-renewal of intestinal tumor cells. Mol Cancer 2017;16:30.
6. Liu YH, Tsang JY, Ni YB, Hlaing T, Chan SK, Chan KF, et al. Doublecortin-like kinase 1 expression associates with breast cancer with neuroendocrine differentiation. Oncotarget 2016;7:1464-76.
7. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, et al. Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet 2013;45:98-103.
8. Weygant N, Qu D, May R, Tierney RM, Berry WL, Zhao L, et al. DCLK1 is a broadly dysregulated target against epithelial-mesenchymal transition, focal adhesion, and stemness in clear cell renal carcinoma. Oncotarget 2015;6:2193-205.
9. Chandrakesan P, Weygant N, May R, Qu D, Chinthalapally HR, Sureban SM, et al. DCLK1 facilitates intestinal tumor growth via enhancing pluripotency and epithelial mesenchymal transition. Oncotarget 2014;5:9269-80.
10. Kadletz L, Thurnher D, Wiebringhaus R, Erovic BM, Kotowski U, Schneider S, et al. Role of cancer stem-cell marker doublecortin-like kinase 1 in head and neck squamous cell carcinoma. Oral Oncol 2017;67:109-18.
11. Li J, Wang Y, Ge J, Li W, Yin L, Zhao Z, et al. Doublecortin-Like Kinase 1 (DCLK1) Regulates B Cell-Specific Moloney Murine Leukemia Virus Insertion Site 1 (Bmi-1) and is Associated with Metastasis and Prognosis in Pancreatic Cancer. Cell Physiol Biochem 2018;51:262-77.
12. Sureban SM, Madhoun MF, May R, Qu D, Ali N, Fazili J, et al. Plasma DCLK1 is a marker of hepatocellular carcinoma (HCC): Targeting DCLK1 prevents HCC tumor xenograft growth via a microRNA-dependent mechanism. Oncotarget 2015;6:37200-15.
13. Whorton J, Sureban SM, May R, Qu D, Lightfoot SA, Madhoun M, et al. DCLK1 is detectable in plasma of patients with Barrett's esophagus and esophageal adenocarcinoma. Dig Dis Sci 2015;60:509-13.
14. Wang YL, Li Y, Ma YG, Wu WY. DCLK1 promotes malignant progression of breast cancer by regulating Wnt/β-Catenin signaling pathway. Eur Rev Med Pharmacol Sci 2019;23:9489-98.
15. Lv Y, Song G, Wang R, Di L, Wang J. Doublecortin-like kinase 1 is a novel biomarker for prognosis and regulates growth and metastasis in basal-like breast cancer. Biomedicine & Pharmacotherapy 2017;88:1198-205.

16. Wang J, Wang S, Zhou J, Qian Q. miR-424-5p regulates cell proliferation, migration and invasion by targeting doublecortin-like kinase 1 in basal-like breast cancer. Biomedicine & Pharmacotherapy 2018;102:147-52.
17. Haakensen VD, Bjøro T, Lüders T, Riis M, Bukholm IK, Kristensen VN, et al. Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas. BMC cancer 2011;11:1-11.